CARBON TETRACHLORIDE DEPRESSES HEPATIC PHOSPHOLIPID SYNTHESIS IN RATS

(Mitochondrial membranes; hepatotoxicity; oxidative phosphorylation)

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SUMMARY

40 h after an acute dose of CCl₄ (11.3 mmol/kg), the incorporation of [1-¹⁴C]ethanolamine into rat hepatic microsomal phospholipids was inhibited to 70% of control. Incorporation into phospholipids of the inner and outer mitochondrial membranes was 30-35% of control. Rates of incorporation were equal to or above normal rates in all membranes 65 h after dosage. The activity of methyltransferase in microsomal fractions isolated from rats 10 to 66 h after dosage was depressed. These data suggest that the alteration of mitochondrial phospholipids that parallels mitochondrial dysfunction after acute CCl₄ dosage could be attributed to a CCl₄-induced inhibition of the microsomal phospholipid biosynthetic pathways.

INTRODUCTION

Mitochondria isolated from rat liver display inhibited rates of respiration and oxidative phosphorylation after an acute dose of CCl₄ [1, 2]. The relative concentrations of phospholipids in the microsomal [3, 4] and both the inner and outer mitochondrial membranes [5] are altered after CCl₄ intoxication. The phospholipid changes in the microsomes precede those in the mitochondrial membranes [5]. The major phospholipids of the mitochondria, PC and PE, are synthesized by enzymes

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Abbreviations: PC, phosphatidylcholine; PD, phosphatidylcholine; PE, phosphatidylcholine; TLC, thin-layer chromatography.
in the endoplasmic reticulum [6, 7] and are transferred to the mitochondria by, as yet, poorly defined mechanisms [8, 9]. Since a profound inhibition of many microsomal enzymes, e.g. glucose-6-phosphatase [10], cytochrome P-450 [11], and the Ca dependent ATPase [12], occurs during the early stages of CCl₄ intoxication, we hypothesized that inhibition of microsomal phospholipid biosynthesis by CCl₄ alters the mitochondrial phospholipid content. The disturbance of normal mitochondrial membrane phospholipid composition could inhibit the activity of the membrane-bound proteins of oxidative phosphorylation, inducing the characteristic lesions of the CCl₄-poisoned hepatic mitochondria. The hypothesis was tested in rats by: (1) determining the in vivo rate of incorporation of [1-³H]ethanolamine into the PE and PC fractions of microsomal and mitochondrial membranes at 40, 65 and 165 h after CCl₄, and (2) measuring the rate of methylation of PE to PC by microsomal enzymes isolated from rat liver 10, 20, 40 and 65 h after an acute dose of CCl₄. The periods correspond to the onset, maximum development, and early resolution of mitochondrial damage after this dose (11.3 mmol/kg) of CCl₄ [1, 13].

METHODS

Animals and treatment

Male Sprague-Dawley rats (Charles River, Portage, MI) were housed in pairs in wire-mesh-bottom cages and maintained on a diet of commercial rat chow (Purina Rat Chow, Ralston-Purina, St. Louis, MO) and water ad lib. Rats were 7-8 weeks old (190-250 g) and fasted overnight prior to killing. Animal quarters were kept on a 12-h light/dark cycle and at a constant temperature (22°C). CCl₄ was administered intraperitoneally (i.p.) without a carrier at 1.1 ml/kg (11.3 mmol/kg) body weight. [1-³H]Ethanolamine HCl (Amersham, Arlington Heights, IL; 2-10 Ci/mmol) was administered i.p. at a dose of 20 μCi per rat.

Preparation of membrane fractions

Rats were killed by decapitation and their livers removed. Livers from 2-10 rats were pooled and minced in ice-cold isolation media (0.25 M sucrose, 10 mM Tris, 10 mM KCl and 1 mM EDTA, pH 7.2). After homogenization, microsomal and mitochondrial subcellular fractions were prepared as previously reported [14] with the addition of a 15 000 x g x 20 min centrifugation of the post-mitochondrial supernatant fraction. This pellet contained both light mitochondria and heavy microsomes and was discarded to optimize membrane purity in the final preparation.

Inner and outer mitochondrial membrane fractions were prepared by the French press method of Decker and Greenawalt [15] with modifications as follows: the first 12 000 x g supernatant fraction containing broken outer membrane fragments was further purified by centrifugation at 27 000 x g for 10 min, which removed fragments of damaged mitochondria, and by a discontinuous sucrose gradient at 77 000 x g for 90 min, to obtain the pellet of outer membrane fragments which was then resuspended in isolation media. Protein was determined by the biuret method [16].
Enzyme assays

Membrane purity was assessed by the following marker enzyme assays: monoamine oxidase for the outer mitochondrial membrane [17], cytochrome c oxidase for the inner mitochondrial membrane [18], and NADPH cytochrome c reductase for the microsomal membrane [19].

The activity of PD methyltransferase was determined by the in vitro incorporation of S-[methyl-^3H]adenosylmethionine (New England Nuclear, Boston, MA; 5–15 Ci/mmol) into microsomal membrane phospholipids [20]. Dipalmitoyl PD (1.75 mM, dissolved in methanol), served as a substrate for the final step in the Bremer-Greenberg pathway for PC synthesis [21].

The entire 3-step methyltransferase pathway was assayed following the same procedure as above with these modifications: sodium deoxycholate was reduced to 0.9 mM, and 2 mg PC was included as an emulsifier. PD was omitted. The rate-limiting step of this pathway is considered to be the first methyl group addition [20, 21].

Phospholipid separation and quantitation

Phospholipids were extracted from microsomal and inner and outer mitochondrial membrane fractions by modification of the method of Folch et al. [22]. After the addition of chloroform–methanol (2:1), samples were shaken for 3 min in a 50°C water bath and then for 20 min at room temperature. More chloroform–methanol (2:1) and 0.4 M KCl were added to each tube. The aqueous phase was separated from the chloroform phase by centrifugation and the latter was pipetted into a clean test tube, evaporated to dryness, and frozen under nitrogen or immediately assayed.

The extracted phospholipids were separated by two-dimensional TLC on Silica Gel G plates (Eastman Kodak, Rochester, NY), and compared to phospholipid standards (Applied Science, Ann Arbor, MI; Sigma Chemical Co., St. Louis, MO). The solvent systems were chloroform–methanol–water–ammonia (70:30:3:2) and chloroform–methanol–water (65:35:5) for the first and second directions, respectively [23]. Spots were visualized with iodine vapour and then scraped into small acid-washed flasks for determination of phosphate content [24].

In vivo synthesis of phospholipids

The synthesis of PE and PC in CCl4-exposed rats was examined by measuring the incorporation of [1-^3H]ethanolamine into phospholipids of microsomal and mitochondrial membranes. Phospholipids were separated by TLC and initially all spots were scraped and counted. Only those spots previously identified as PE and PC contained any label.

Two control and two 40-h CCl4 animals were injected with 20 μCi [1-^3H]ethanolamine at 1, 2 and 3 h prior to killing to determine the time of maximum label incorporation in the microsomal and mitochondrial membrane phospholipids. In the microsomal phospholipids the greatest amount of label was found 2 h after dosing.
TABLE I

<table>
<thead>
<tr>
<th>Membrane fraction</th>
<th>Time after [3H]ethanolamine (h)</th>
<th>Radioactivity (cpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Microsomal</td>
<td>1</td>
<td>4531</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5059</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4036</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>1</td>
<td>886</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1252</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1418</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCl₄</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1131</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2931</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1998</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>304</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>872</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>697</td>
</tr>
</tbody>
</table>

in both control and 40-h CCl₄ animals (Table I). The difference in label incorporation into mitochondrial membranes between the 2- and 3-h time points was small. Therefore, in all further experiments the specific radioactivities of PC and PE were determined after extraction of the lipids from animals killed 2 h after receiving the [1-3H]ethanolamine. Considerably less label (25–70%) was present in all CCl₄ fractions than in the corresponding control sample (Table I).

Statistical significance

Significance was determined by analysis of variance and comparison of means by the Bonferroni-Scheffe method [25]. Significance was assumed for $P<0.05$.

RESULTS

Purity of the membrane fractions was assessed by determination of marker enzyme activities and did not differ markedly from control at any time after CCl₄ exposure except for an increased inner mitochondrial membrane contamination of the outer membrane fraction 40 h after CCl₄. Based on the specific activity of cytochrome c oxidase in the outer membrane fraction, inner membrane contamination of outer membrane protein increased from 18 to 46% at 40 h. The effect of this contamination was to dilute the extent of changes observed in the outer membrane.

Incorporation of [3H]ethanolamine at 40 h into outer membrane and inner membrane PE was <30 and 55% of control, respectively. Microsomal PE-specific radioactivity was 67% of control levels at this time (Table II). The specific radioactivity of PE returned to control levels in the microsomal and inner membrane fractions by 65 h after CCl₄, but the specific radioactivity of PE in the outer membrane fraction was twice that observed in controls. The rate of PE synthesis in all three
TABLE II

[3H]ETHANOLAMINE HCl INCORPORATION IN MICROSOMAL AND MITOCHONDRIAL PHOSPHATIDYLCHOLINE (PC) AND PHOSPHATIDYLETHANOLAMINE (PE) FOLLOWING CCl4 INTOXICATION

Phospholipids were extracted from inner and outer mitochondrial and microsomal membrane fractions as described in METHODS. Incorporation is expressed as CPM/μg phospholipid-phosphorus. Numbers in parentheses are percent control. Fractions were prepared from 10 rats at each time point.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control</th>
<th>Time after CCl4 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>45.5</td>
<td>14.0 (31) 65.3 (144) 50.3 (111)</td>
</tr>
<tr>
<td>PE</td>
<td>337.5</td>
<td>97.8 (29) 683.1 (202) 384.1 (114)</td>
</tr>
<tr>
<td>Inner mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>32.7</td>
<td>13.4 (39) 59.5 (182) 42.3 (127)</td>
</tr>
<tr>
<td>PE</td>
<td>288.3</td>
<td>156.1 (54) 268.6 (93) 217.1 (75)</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>59.9</td>
<td>33.1 (55) 73.5 (123) 58.0 (97)</td>
</tr>
<tr>
<td>PE</td>
<td>722.2</td>
<td>483.0 (67) 717.1 (99) 713.0 (99)</td>
</tr>
</tbody>
</table>

TABLE III

EFFECT OF CCl4 INTOXICATION ON THE IN VITRO METHYLTRANSFERASE ACTIVITY OF THE MICROSOMAL MEMBRANE FRACTION FROM RAT HEPATOCYTE

Microsomal membrane fractions were prepared from individual livers. Activity is expressed as CPM of [3H]S-adenosylmethionine incorporated per mg protein per 15 min incubation at 37°C. The synthesis of PC from PE (PE→PC) and from phosphatidyldimethylethanolamine (PD→PC) was measured (see METHODS).

<table>
<thead>
<tr>
<th>Time after CCl4 (h)</th>
<th>PE→PC</th>
<th>PD→PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>412.8 ± 47.3 (8)</td>
<td>115.1 ± 10.9 (8)</td>
</tr>
<tr>
<td>10</td>
<td>192.3 ± 29.9 (4)b</td>
<td>50.6 ± 18.4 (5)b</td>
</tr>
<tr>
<td>20</td>
<td>284.4 ± 43.4 (8)</td>
<td>60.6 ± 9.5 (7)b</td>
</tr>
<tr>
<td>40</td>
<td>226.3 ± 33.6 (4)</td>
<td>57.3 ± 16.1 (4)b</td>
</tr>
<tr>
<td>65</td>
<td>212.5 ± 44.9 (2)</td>
<td>61.5 ± 9.6 (6)b</td>
</tr>
</tbody>
</table>

Significance (determined by one-way analysis of variance and Bonferroni-Scheffe analysis of means): aP<0.05; bP<0.01.

membrane fractions at 165 h was essentially that of control values.

The pattern of changes of PC synthesis 40 h after CCl4 was similar to that of PE. Incorporation of [3H]ethanolamine into PC in the microsomal membrane was 55% of control, while the labelling of PC in both mitochondrial membranes was depressed 30–41% at this time. All three fractions had elevated rates of labelling 65 h after CCl4. The labelling of PC in mitochondrial membranes remained above control values at 165 h.
Methyltransferase activity was measured in microsomes isolated from CCl₄-dosed rats (Table III). As expected, the 3-step methylation pathway (PE to PC) incorporated roughly 3 times the radioactivity as did the final step (PD to PC). The specific activity of the 3-step methylation pathway of PE to PC in vitro was depressed at 10 h after in vivo exposure to CCl₄ (Table III). A significant decline in activity was found at all times when the methylation of PD was assayed. In both assays, the greatest depression of activity was found at the earliest time examined, 10 h after CCl₄ exposure. Methyltransferase activity was not detected in the mitochondrial fraction from either control or CCl₄-exposed rat livers.

DISCUSSION

Reduced levels of newly synthesized PE and PC occur in the hepatic microsomal and inner and outer mitochondrial membrane fractions 40 h after CCl₄, the same time that functional and compositional alterations are found in these membranes. Rates of oxidative phosphorylation are severely inhibited [1, 2] and evidence of outer-membrane damage is present [13]. Similar studies reported that cytochrome P-450 concentrations [11], glucose-6-phosphatase activity [10, 11] and Ca-ATPase activity [12] are depressed in microsomal fractions from rats after CCl₄ exposure. The depressed rates of synthesis of PE and PC are consistent with previously described changes of phospholipid content in microsomal and mitochondrial membranes [5] and suggests that the altered phospholipid composition of these membranes could be attributed, at least in part, to impaired function of the enzymes involved in phospholipid synthesis. At 65 h after CCl₄, when mitochondrial function and phospholipid composition are partially restored to normal [1, 5] the specific activity of PC was greater than that of control in all 3 of the membrane fractions and that of PE was increased in the outer-membrane fraction (Table II). This is consistent with an accelerated rate of synthesis of membrane components associated with the restoration of mitochondrial function at 65 h.

The enzymes involved in the methylation of PE to PC were significantly depressed 10 h after CCl₄. The depressed activity of the PD methyltransferase pathway at 65 h appears to contradict the increased amount of labelled PC present in the microsomes at this time as detected by the in vivo experiment. Apparently, the in vitro methyltransferase activities were sufficient to fulfill the in vivo demands of this pathway.

The critical processes by which CCl₄ produces hepatocyte death are not resolved [26]. However, the association between proper membrane function and lipid composition is well recognized [4, 27]. These results suggest that the inhibition of phospholipid synthesis may be important in the development of membrane damage and that recovery of synthesis is concomitant with the resolution of damage.
ACKNOWLEDGEMENT

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REFERENCES